

DESCRIPTIONMethods and Devices for Quantitation of Glycated ProteinPriority Information

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This application claims priority to United States Application Serial No. 60/265,229, filed January 31, 2001.

Field of the Invention

10 This invention relates to a method for the quantitation of percent glycated protein in a biological sample suitable for use with a reflectance meter such as used in the self monitoring of blood glucose concentration by diabetics.

Background of the Invention

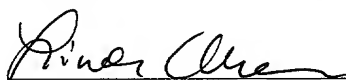
15 Control of blood glucose concentrations in diabetics has been shown to decrease the frequency and severity of long-term microvascular and neurologic complications of the disease. The measurement of glycated hemoglobin and protein in blood are used to determine how well blood glucose concentration has been managed over an extended time period.

20 The rate of formation of glycated hemoglobin is directly related to the glucose concentration in blood.

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The average red blood cell life span is 120 days, so quantitation of the percent glycation of hemoglobin has been correlated to a measure of the average glucose concentration over the previous 2 to 3 months which is a measure of glycemic control over that time period (see "Diabetes Control and Complications Trial Research Group, The effect of intensive treatment of diabetes on the development of progression of long-term complications in insulin-dependent diabetes mellitus", New England Journal of Medicine, 329, 977-986 (1993), and "American Diabetes Association, Tests of Glycemia in Diabetes", Diabetes Care, 20 (suppl. 1), S18-S20 (1977)).

Glucose also attaches to non-hemoglobin proteins in blood, for example albumin. Since albumin is the most abundant serum protein and its circulating half-life is about 20 days, the concentration of glycated protein is a measure of the average glucose concentration over the previous 2 to 3 weeks. The measure of glucose directly gives the glucose concentration at the time of measurement.

An immobilized dihydroxyboryl compound has been reported as useful to bind to the 1,2 cis diols of the carbohydrate of glycated proteins to separate them from non-glycated proteins. Use of this technology in a column chromatography method to determine percent glycation has been reported (see, U.S. Pat. No. 4,269,605 issued May 26, 1981 to Dean and U.S. Pat. No. 5,284,777, issued February 8, 1994 to Rosenthal). These methods are said to use the boronate derivative immobilized onto agarose beads in a column to separate glycated from non-glycated proteins in the sample. These methods require specific dilutions and pipettings of the sample so as to not overload the capacity of the affinity binder affixed to the agarose beads. The use of a boronate derivative immobilized on

agarose beads would not appear to lend itself to a strip application.

U.S. Pat. No. 5,110,745 issued May 5, 1992 to Kricka, et al., is said to describe methods of detecting glycated protein in a sample wherein the sample is contacted with a defined excess of a boronate compound in solution. The resulting unbound boronate is said to be measured by binding it to an immobilized glycated molecule on a support matrix and measuring the amount of glycated molecule left un-complexed. This method appears to require a number of steps including, a separate reaction in solution before application to a solid support, dilution of the sample to assure that the amount of binder added to the biological sample is in excess, performance of a separate assay to determine the percentage of protein glycation and multiple binding and washing steps.

A dipstick method for the measurement of glycated hemoglobin is said to be described in U.S. Pat. No. 4,861,728 issued August 29, 1989 to Wagner. This method is said to involve contacting of a hemoglobin binding agent linked to a solid support with a lysed blood sample previously mixed with a dihydroxyboryl compound linked to a fluorescent label. The support is said to bind non-glycated hemoglobin and fluorescent labeled glycated hemoglobin. The fluorescent label is said to bind to glycated hemoglobin through the dihydroxyboronyl compound. The solid phase is removed from the sample and total hemoglobin is measured by reflectance photometry while glycated hemoglobin is measured using fluorescence. This method is said to require an addition of an amount of fluorescent labeled dihydroxyboryl reagent to the sample and a rinsing step after it is removed from the sample.

Further it requires two different measurement methods for the quantitation.

In other assays, (see, e.g., Japanese Pat. No. 6,058,936, European Pat. No. 455225, and published PCT application WO 96/03657) a boronate derivative coupled to a detectable label (such as a fluorescent compound, a chemiluminescent compound, isotope, enzyme or other label) is said to be used. Both the glycated and non-glycated proteins are bound to a solid support using a general affinity binder such as an antibody. The boronate-label complex is added and the amount of label that remains bound to the solid support is measured. Each of these types of methods requires the additional step of labeling the glycated protein. In addition, these assays use different measurement methods to quantitate total and glycated proteins.

Published PCT application WO 9840750 (published September 17, 1998) is said to describe a method of determining the percentage of glycated hemoglobin in which immobilized boronate binds glycated hemoglobin in the sample. The amount of glycated hemoglobin bound is said to be proportional to the fraction of glycated hemoglobin in the sample. This is said to eliminate the need for measuring nonglycated hemoglobin to determine the percent glycation. However, it appears the method results may vary depending on the incubation time of glycated protein with the boronated support.

Consequently, there is a need for a simple, fast and efficient method to quantitate the amount of glycated protein in a biological sample that does not require dilution of the sample, requires minimal procedural steps, may be utilized in conjunction with a simple detection

device such as a hand held reflectance meter, and is adaptable to a standard strip assay.

Summary of the Invention

The present invention is directed to methods for the quantitation of glycated proteins in a biological sample, devices utilizing these methods and kits comprising these devices.

The results from measurement of glucose, glycated protein and glycated hemoglobin may provide a more complete picture of glycemic control. Measurement of immediate glucose concentration may be used for adjustment of medications, diet or exercise. For measurement of medium term glycemic control, measurement of glycated albumin concentration may allow one to follow effects of recent changes in lifestyle. For measurement of long term glycemic control, measurement of glycated hemoglobin concentration may allow one to monitor overall effects of changes. It would be convenient to have a test for all three in a format that could be used in a doctor's office lab so results could be discussed with the patient during the visit or, alternatively, used by the patient at home. Currently, blood glucose is routinely monitored using hand held meters and easy to use strips. Unfortunately, similar tests for glycated proteins and hemoglobin have not been available.

The methods of the present invention provide methods of quantitating glycated protein in a biological sample. The biological sample is contacted with a solid support matrix under conditions where both glycated and non-glycated protein are bound to the solid support matrix. An amount of a first buffer is added sufficient to rinse off unbound protein. A first bound protein measurement is made

to determine total (glycated and non-glycated) bound protein. A second buffer is added to the solid support matrix which changes the conditions so that glycated protein is bound and non-glycated protein is not substantially bound and is added in an amount sufficient to rinse off unbound (non-glycated) protein. A second bound protein measurement is made. Glycated protein is quantitated using the first and second bound protein measurements.

According to one aspect, the present invention is directed to a method of quantitation of glycated protein in a sample which comprises: (a) contacting a solid support matrix which comprises a negatively charged group and a hydroxyboryl compound and which has a measurement area, with an aliquot of biological sample sufficient to cover said measurement area; (b) contacting said solid support matrix with an aliquot of a first buffer sufficient to rinse off unbound protein, wherein said first buffer has a pH selected to allow both glycated and non-glycated protein to be bound to said solid support matrix; (c) quantitating protein bound to said measurement area using measurement of a selected property of said protein to give a first bound protein reading; (d) contacting said solid support matrix with an aliquot of a second buffer sufficient to rinse off unbound protein, wherein said second buffer has a pH selected to allow glycated protein to be bound to said solid support matrix but where non-glycated protein is not substantially bound to said solid support matrix; (e) quantitating protein bound to said measurement area using measurement of the property measured in step (c) to give a second bound protein reading; and (f) calculating percentage of glycated protein using said first and second bound protein readings.

According to an alternate aspect, the present invention is directed to a method for quantitation of amount of glycated protein in a biological sample which compromises: (a) contacting a solid support matrix which
5 comprises a negatively charged group and a hydroxyboryl compound and which has a measurement area with an aliquot of a biological sample sufficient to cover said measurement area; (b) contacting said solid support matrix with an aliquot of a first buffer sufficient to rinse off unbound
10 protein, wherein said first buffer has a pH of about 5.0 to about 7.0; (c) quantitating protein bound to said measurement area to give a first bound protein reading; (d) contacting said solid support matrix with an aliquot of second buffer sufficient to rinse off unbound protein,
15 wherein said buffer has a pH of about 8.0 to about 10.0; (e) quantitating protein bound to said measurement area to give a second bound protein reading; and (f) calculating percentage of glycated protein in said sample using said first bound protein reading and said second bound protein
20 reading.

Preferably, and for convenience, the first and second bound protein readings measure the same property. Preferably, the property measured is an optical reading. More preferably, the optical reading is absorbance or
25 reflectance at a specified wavelength.

According to one preferred aspect of the present invention, a method for quantitation of glycated hemoglobin in a biological sample is provided which comprises:
(a) bringing the biological sample into contact with a
30 solid support matrix which comprises negatively charged groups and a dihydroxyboryl compound and which has a measurement area, at a sample application site which is in communication with the solid support matrix; (b) adding an

aliquot of a first buffer at the sample application site wherein the first buffer has a pH of about 5.0 to about 7.0; (c) making a first optical reading of said measurement area at a wavelength at which hemoglobin absorbs light; (d) adding an aliquot of a second buffer at the sample application site wherein the second buffer has a pH of about 8.0 to about 10.0; (e) making a second optical reading of the measurement area at a wavelength at which hemoglobin absorbs light; and (f) calculating the percentage of glycated hemoglobin in the blood sample using the first and second optical readings. Where the biological sample is a blood sample comprising red blood cells, the sample is contacted with a red blood cell lysing agent before the first optical reading is made. The blood sample may be pre-treated with a red blood cell lysing agent prior to being brought into contact of the solid support. Alternatively, the first buffer may further comprise a red blood cell lysing agent or the solid support matrix may be treated with a red blood cell lysing agent before it comes in contact with the biological sample.

According to another preferred aspect of the present invention, a method for quantitation of a glycated non-hemoglobin protein is provided which comprises: (a) bringing the biological sample into contact with a sample application site which is in communication with a solid support matrix which comprises negatively charged groups and a dihydroxyboryl compound and which has a measurement area; (b) adding an aliquot of a first buffer to the sample application site wherein the first buffer has a pH between about 5.0 and about 7.0; (c) making a first optical reading of the measurement area at a wavelength at which the protein absorbs light; (d) adding an aliquot of a second buffer to the sample application site wherein the second

buffer has a pH between about 8.0 and about 10.0; (e) making a second optical reading of the sample application site at a wavelength at which the protein absorbs light; and (f) calculating the percentage of glycated protein in the biological sample using the first and second optical readings. Suitable biological samples for use in the method of this aspect of the present invention include plasma and serum samples. A suitable glycated protein for quantitation according to this aspect is albumin. For the quantitation of certain glycated proteins it may be preferred that the protein be labeled with a suitable protein specific labeling agent.

Preferred buffer systems for use as the first buffer include MES [2(N-morpholino) ethanesulfonic acid], MOPS [3(N-morpholino)propanesulfonic acid] and HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid].

Preferred buffers for use as the second buffer include ammonium acetate and taurine buffers.

According to another aspect of the present invention, a diagnostic device for quantitation of glycated protein utilizing the methods above is provided. Preferably the protein is hemoglobin or albumin.

In another aspect, a kit is provided which comprises the diagnostic device described above; a first buffer having a pH of about 5.0 to about 7.0; and a second buffer having a pH of about 8.0 to about 10.0.

Definitions

In accordance with the present invention and as used herein, the following terms are defined to have the following meanings, unless explicitly stated otherwise:

The term "alkyl" refers to saturated aliphatic groups including straight-chain, branched-chain and cyclic (including polycyclic) groups.

5 The term "carboxylate" or "carboxy" refers to the group -COOH.

The term "phosphate" refers to the group - PO₄.

The term "sulfate" refers to the group -SO₄.

The term "sulfinate" refers to the group -SO₂H.

The term "sulfonyl" refers to the group -SO₃H.

10 "Hb" refers to hemoglobin.

"HEPES" refers to [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid].

15 "K", when used in the context of measurement of amount of light absorbed and reflectance, refers to the absorption coefficient.

"MES" refers to [2(N-morpholino)ethanesulfonic acid].

"MOPS" refers to [3-(N-morpholino)propanesulfonic acid].

20 "S", when used in the context of measurement of amount of light absorbed and reflected, refers to the scattering coefficient.

Brief Description of the Drawings

25 Figure 1 depicts a graph of the absorbance of glycated hemoglobin (closed triangles) and total hemoglobin (open triangles) bound to the solid support in the assay and a graph of the ratio of glycated to total hemoglobin (open circles) versus soak time. Different supports were used, having been derivatized with boronate for increasing lengths of time.

Figure 2 depicts a graph of optical absorbance of bound hemoglobin at 415 nm vs. incubation time for blood lysate of a diabetic sample comparing a single measurement assay ("X" for glycated) to the method of the present invention (circles for total Hb, open triangles for glycated Hb).

Figure 3 depicts a graph of optical absorbance at 415 nm of glycated hemoglobin or fraction of glycated hemoglobin vs. incubation time for blood lysate of a diabetic sample comparing a single measurement assay (adapting the method of published PCT application WO 98/40750 "X") to the method of the present invention (open triangles).

Figure 4 depicts a graph of the absorbance of hemoglobin binding to the carboxy cellulose support (no added boronate groups) over a pH range.

Figure 5 depicts a graph of the absorbances of hemoglobin (closed circles) and of albumin (open triangles) bound to the boronated support over a pH range.

Figures 6A, 6B and 6C depict three strip configurations which may be used according to the methods of the present invention.

Figure 7 depicts a graph of K/S vs. percent glycated hemoglobin ("X") showing proportionality of present invention assay results to the concentration of glycated hemoglobin in the sample in a strip assay format using the method of the present invention (open triangles depict total hemoglobin).

Figure 8 depicts a graph of K/S fraction of glycated hemoglobin (glycated hemoglobin divided by total hemoglobin) vs. percent glycated hemoglobin showing proportionality of the fraction to glycated hemoglobin

concentration in the sample in a strip assay format using the method of the present invention.

Figure 9 depicts a graph of optical absorbance at 280 nm for total (open triangles) and glycated albumin ("X") vs. relative fructosamine content (proportional to fractional amount of glycated albumin) in a sample.

Figure 10 depicts a graph of the ratio of glycated albumin to total albumin (absorbance at 280 nm) vs. relative fructosamine content (proportional to the fractional amount of glycated albumin) in a sample.

Figure 11 depicts a graph of optical absorbance at 415 nm of bound total hemoglobin (closed triangles) and glycated hemoglobin (open triangles) vs. whole blood sample volume in a lateral flow device. (See Figure 6A)

Figure 12 depicts a graph of the ratio of glycated hemoglobin divided by total hemoglobin vs. whole blood sample volume in a lateral flow device. (See Figure 6A)

Figure 13 depicts a graph of optical absorbance at 415 nm of total hemoglobin (open triangles) and glycated hemoglobin ("X") vs. dilution of a normal blood sample lysate.

Figure 14 depicts a graph of optical absorbance at 415 nm of total hemoglobin (open triangles) and glycated hemoglobin ("X") vs. dilution of a diabetic blood sample lysate.

Figure 15 depicts a graph of optical absorbance at 415 nm of glycated hemoglobin divided by total hemoglobin vs. dilution of a normal ("X") and diabetic blood (open triangles) sample lysate.

Figures 16A and 16B depict graphs showing correlation between whole blood assay results using an assay of the present invention and two other commercially available

assays for hemoglobin A1c. Figure 16A depicts a plot of results obtained using an assay of the present invention (See Example 9) versus results obtained using the Roche® Integra 400 (Roche Diagnostics). Figure 16B depicts a plot of results obtained using an assay of the present invention (See Example 9) versus results obtained using the DCA 2000 (Bayer).

Detailed Description of the Invention

According to one aspect, the present invention provides a method for quantitation of glycated proteins in a biological sample in which the sample and reagent(s) are applied to a solid support matrix which preferably comprises a negatively charged group and a dihydroxyboryl compound. Measurement of the total and glycated proteins is done at a single location on the solid support. This measurement may conveniently be done by measuring a selected property of the protein. Advantageously, this method does not require measurement of the volume of biological sample.

In one aspect, the present invention is directed to a method for quantitation of glycated protein in a sample in which a solid support matrix which comprises a negatively charged group and a hydroxyboryl compound and which has a measurement area is contacted with an aliquot of the biological sample sufficient to cover the measurement area. The solid support matrix is then contacted with a first buffer which has a pH selected so as to allow both glycated and non-glycated protein to be bound to the solid state matrix, in an amount sufficient to rinse off unbound protein. The amount of protein bound to the measurement area is quantitated by measurement of a selected property of the protein to give a first bound protein reading. The

solid support matrix is then contacted with a second buffer which has a pH selected to allow glycated protein to be bound to the solid support matrix but where non-glycated protein is not substantially bound to the solid support
5 matrix, in an amount sufficient to rinse off unbound protein. The amount of protein bound to the measurement area is quantitated by measurement of the same selected property (as used to obtain the first bound protein reading) to give a second bound protein reading. The
10 percentage of glycated protein is calculated using the first and second bound protein readings.

According to an alternate aspect, the present invention is directed to methods of quantitation of glycated protein in a sample in which a solid support
15 matrix which comprises a negatively charge group and a hydroxyboryl compound and which has a measurement area is contacted with an aliquot of a biological sample sufficient to cover the measurement area. The solid support matrix is then contacted with a first buffer which has a pH of about
20 5.0 to about 7.0 in an amount sufficient to rinse off unbound protein. The amount of protein bound to the measurement area is quantitated to give a first bound protein reading. The solid support matrix is then contacted with a second buffer which has a pH of about 8.0
25 to about 10.0 in an amount sufficient to rinse off unbound protein. The amount of protein bound to the measurement area is quantitated to give a second bound protein reading. The percentage of glycated protein in the biological sample is calculated using the first bound protein reading and the
30 second bound protein reading.

The methods of the present invention may conveniently be used to quantitate amounts of either glycated hemoglobin or glycated non-hemoglobin protein (such as albumin).

In the quantitation of glycated hemoglobin, typically a blood sample is used. The blood sample is contacted with a red blood cell lysing agent before the first bound protein reading is made. The sample may be contacted with the red blood cell lysing agent prior to being added to the solid support (such as by pretreatment of the sample). Also, the red blood cell lysing agent may be added to the solid support by a pre-rinse before sample is added to the support. Alternatively, the first buffer may include a red blood cell lysing agent. Suitable red blood cell lysing agents are known to those of skill in the art and include Triton X-100 and Igepal CA-630.

Preferably, the first and second bound protein readings involve an optical reading. More preferably the selected property measured is absorbance or reflectance at specified wavelength.

According to a preferred aspect of the present invention, the dihydroxyboryl compound of said solid support matrix has the structure;



wherein R is selected from the group consisting of phenyl, substituted phenyl, hydrogen, and alkyl of 1 to about 6 carbon atoms. When R is alkyl, suitable alkyl groups include ethyl, 1-propyl, and 3-methyl-1-butyl. Preferably R is m-amino-phenyl.

Suitable solid support matrices include supports selected from the group consisting of cellulose, nitrocellulose, cellulose acetate, polyacrylamide, agarose polyacrylamide copolymer, agarose, starch, nylon, nylon polyesters, dextran, cross-linked dextran, dextran

acrylamide copolymer, cross-linked hydroxyethylmethacrylate substituted cross-linked polystyrenes, polyvinylalcohol, wool, metal oxides, porous ceramics coated with hydrophilic organic polymers and glass. Preferably the solid support
5 matrix comprises a negatively charged group. A preferred solid support matrix is cellulose. Preferably the negatively charged group is selected from the group consisting of phosphate, sulfate, sulfonate, sulfinic and carboxylate (or carboxy). Preferably the negatively
10 charged group is carboxylate.

I The Assay

According to one aspect of the method of the present invention both glycosylated and non-glycosylated protein are bound to a solid support matrix comprising a negatively charged
15 group and an immobilized dihydroxyboryl containing compound under one pH condition. The bound non-glycosylated protein is then selectively removed under a second pH condition. The glycosylated protein remains bound to the support under the second pH condition. The measurement of total protein is
20 made following the binding under the first pH condition. The measurement of glycosylated protein is made after rinsing the support-bound protein complex under the second pH condition to remove unbound protein. The percent glycosylation is calculated from the percent of the second measurement
25 relative to the first. This assay is particularly useful for the measurement of glycosylated hemoglobin and glycosylated protein in a blood, serum or plasma sample.

The method may be used for a biological sample such as capillary blood, whole blood, serum or plasma. According
30 to a preferred aspect, one wavelength may be used for both measurements (total and glycosylated). The assay is easily adaptable to be used with the same meters that are used in

self blood glucose monitoring by diabetics. Since both measurements may be made at the same location on the solid support (see Example 5), any differences in the support from location to location would not interfere with the results.

II Solid Support Matrix and Immobilized Binder

The solid support matrix may be one of a number of natural or synthetic polymeric materials to which can be bound negatively charged groups and the dihydroxyboryl compound and through which the sample and reagents can pass. Suitable support matrices include, for example, cellulose, nylon, nitrocellulose cellulose acetate, polyacrylamide, agarose polyacrylamide copolymer agarose, starch, nylon polyesters, dextran, cross-linked dextran, dextran acrylamide copolymer, cross-linked hydroxyethylmethacrylate substituted cross-linked polystyrenes and polyvinyl alcohol. Other supports that may be utilized with the present invention include those listed in U.S. Pat. No. 4,269,605. In addition, other suitable supports are known to those of skill in the art. A preferred solid support matrix is cellulose paper.

The dihydroxyboryl compound (preferably phenylboronic, boric or other boronic acids, more preferably m-aminophenylboronic acid) may be bound to the solid support by mechanical, physical or chemical means, preferably by a covalent chemical bond. Binding to the solid support matrix can be by methods known in the art. Such methods include, for example, those listed in U.S. Pat. No.: 4,269,605 to Dean et al, (issued May 26, 1989).

After binding the dihydroxyboryl compound to the support, the support can be rinsed with buffer or water. If the biological sample to be assayed has red blood cells,

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a detergent or other red blood cell lysing agent may optionally be included in the rinse if the assay is to measure glycated hemoglobin. Suitable detergents for use as a red blood cell lysing agent include Triton X-100. The
5 presence of lysing agent in the support may facilitate the lysing of red blood cells from a whole blood sample. Alternatively, the sample may be pretreated with the red blood cell lysing agent or the red blood cell lysing agent may be included in the first buffer.

10 III Configuration of Solid Support Matrix

According to one aspect of the present invention, the solid support matrix is placed in a strip-type configuration along with other components to handle the flow of sample and buffer. The sample and buffers flow
15 through the solid support matrix where binding occurs, and then into an absorbent material which will soak up the excess solution, allowing adequate volumes of buffer to pass through. Flow of solution through the strip can be along the length of the solid support matrix as in a
20 lateral flow device, or through the thickness of the solid support, as in a vertical flow device (examples of such strip configurations are depicted in Figures 6A, 6B and 6C).

Suitable materials for use in the solid support
25 include, for example, cellulose (including cellulose paper), nitrocellulose, cellulose acetate, polyacrylamide, agarose polyacrylamide copolymer, agarose, starch, nylon, nylon polyester, dextran, cross-linked dextran, dextran acrylamide copolymer, cross-linked hydroxyethylmethacrylate
30 substituted cross-linked polystyrenes, polyvinylalcohol, wool, metal oxides, porous ceramics coated with hydrophilic organic polymers and glass.

IV The Sample and Preferred Buffers

Suitable biological samples for use according to the methods of the present invention include whole blood, serum and plasma. Red blood cells can be removed from the sample if desired by use of methods described in the art.

Included are such methods using glass fibers (U.S. Pat. No. 4,477,575 issued 10/16/84 to Vogel et al.), carrier containing carbohydrate (U.S. Pat. No. 4,678,757 issued 7/7/87 to Rapkin) or a matrix containing a polyol (U.S. Pat. No. 5,725,774 issued 3/10/98 to Neyer).

According to a preferred aspect (especially for quantitation of non-hemoglobin glycated protein), the sample can be pretreated with a protein specific binding agent such as "dye" to label proteins and which may facilitate measurement of their concentration. A variety of such dyes known to those skilled in the art may be utilized with the method of the present invention. For example, a fluorescent dye such as fluorescein isothiocyanate ("FITC") may be used.

The sample is added to the solid support and allowed to flow through the support. The flow can be through the length of the support or through the thickness. The volume of sample added can range from a volume just large enough to cover the measurement area uniformly (such as about 3 to 5 μ L) to a volume that will pass through the support in a reasonable time (approximately 40 μ L). The actual volume limits will depend on the dimensions of the solid support and the other strip components. A larger (wider or longer) strip will be able to handle larger volumes of sample, but will require larger minimum volumes to cover the measurement area. A smaller strip will function with small volumes, but will not be able to handle large volumes.

After the sample has been absorbed into the strip, the first buffer is added. The pH of the first buffer is preferably between about 5.0 and about 7.0. More preferably, the pH of the first buffer is about 5.5 to about 7.0 for quantitation of glycated hemoglobin and about 5.0 to about 6.5 for quantitation of glycated hemoglobin. The buffer contains a suitable buffering agent which has a pKa appropriate for control of the pH within the given range, but that does not otherwise affect the binding of the protein. Buffering agents suitable for use in the first buffer are known to those of skill in the art. Preferred buffering agents for use in the first buffer include MES, MOPS and HEPES. The buffering agent is present in a concentration sufficient to maintain the pH in the desired range. Suitable concentrations for the buffering agent may range from about 5mM to about 500mM. If the biological sample includes red blood cells, the buffer may further comprise a cell lysing agent such as, for example, Triton X-100 or Igepal CA-630. The buffer volume added can be standardized to a specific number of drops adequate to rinse through excess, non-bound sample. The adequacy of rinsing can also be monitored by the reflectance meter taking multiple measurements with the final measurement taken when the change is essentially zero.

The second buffer preferably has a pH from about 8.0 to about 10.0. The second buffer contains a suitable buffering agent which has a pK appropriate for control of pH within the given range, but that does not interfere with the binding of glycated protein to the immobilized dihydroxyboronate compound. Buffering agents suitable for use in the second buffer are known to those of skill in the art. Preferred buffering agents for use in the second

buffer include ammonium acetate and taurine. The buffering agent is present in a concentration sufficient to maintain the pH in the desired range. Suitable concentrations for the buffering agent may range from about 5mM to about 500mM, or, alternatively, up to the solubility limit of the buffering agent. The adequacy of rinsing can be monitored as described for the first buffer. Optionally, the buffer can contain a divalent metal ion such as Mg^{++} to help stabilize the binding of the protein to the boronate.

10 V Quantitation

The amount of protein bound to the support after each rinse can be quantitated using measurement methods known to those skilled in the art including measurement of optical absorbance or reflectance at an appropriate wavelength.

15 According to a preferred aspect of the method, a first reflectance measurement is made of the solid support after sample is added and the excess is rinsed through using the first buffer. This reflectance measurement is used in calculating the total protein concentration using an algorithm derived from calibration data or using "K/S" values as values proportional to concentration. A second reflectance measurement is made after washing off the non-glycated protein from the solid support using the second buffer. This measurement is used to calculate the glycated protein concentration in the same way as the first measurement. The percent glycated protein is calculated as the ratio of the glycated protein concentration to the total protein concentration x 100.

When light enters a transparent media, some of it is absorbed and some passes through the media. The Beer-Lambert law provides that the amount of light absorbed ("A") is the product of the concentration of the material

absorbing the light ("c") and the length the light travels through the media ("d") (i.e. $A=ecd$, wherein e is the extinction coefficient of the absorbing material). When light enters a solid, the amount that reflects off the solid is determined by what is absorbed by the solid and what is scattered by the solid. The model used to define this relationship is the Kubelka-Munk Theory. This model defines two differential equations for the change in the light intensity as it passes into the solid and as it is scattered back out of the solid. The equations include the symbol "K" which is the absorption coefficient in solid phase and the symbol "S" which is the scattering coefficient. In the simplified derivation, the equations reduce to the following: $K/S = [(1-R)^2]/(2R)$ where R is the measured reflectance and K/S is proportional to the concentration of the absorbing/scattering material. In certain of the Figures (see, e.g., Figures 7 and 8) the data taken using the reflectance meter are plotted with y values of K/S which are proportional to the concentration of the analyte.

The methodology used in the assay is adaptable for use in a strip that can be used with hand held reflectance meters used in glucose assays.

The total amount of protein that binds to the solid support is controlled by the binding capacity of the negatively charged groups immobilized on the support. We have observed that glycated and non-glycated protein rapidly bind to the derivatized support in the same ratio as in the sample. The volume of sample added to the support does not have to be measured since any excess will be rinsed through.

The reported percent glycation is calculated from the ratio of two measurements made at the same location on the

solid support in the strip. Therefore, variations in the total amount of protein bound by different strips will not affect the reported result. Since both total and glycated protein are measured using the same method at the same
5 location on the solid support, variations which could affect the measurements will have the same effect on both measurements, minimizing "noise".

To assist in understanding, the present invention will now be further illustrated by the following examples.
10 These examples, as they related to the present invention, should not, of course, be construed as specifically limiting the invention. Also, such variations of the invention, now known or later developed, which would be within the purview of one skilled in the art are considered
15 to fall within the scope of the present invention, as described herein and herein after claimed.

Examples

Example 1

Preparation of the Boronated Solid Support Matrix

20 According to a preferred aspect of the detection method of the present invention, a solid support matrix suitable for use in a strip configuration is prepared by covalently attaching a derivative of boronate, m-aminophenylboronate to a carboxy cellulose based material
25 using a conventional linking chemistry.

Materials

1. Solid Support Matrix: cellulose based solid with carboxylic acid groups covalently bound (Sartobind C membrane, Sartorius).

2. EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) (Pierce)
3. Boronate derivative: m-aminophenylboronic acid (Sigma-Aldrich)
- 5 4. Buffer: 0.1M MES buffer, pH 6.5

Procedure

A 46.7 mg portion of m-aminophenylboronate is dissolved in 25 mL of 100 mM MES buffer. The pH is re-adjusted to 6.5 after the boronate dissolves. A 28.9 mg
10 portion of EDC is dissolved in the MES-boronate solution. The solid support matrix is soaked in the solution for the desired time (25 mL of solution was enough to treat a 30 cm² piece of matrix). The matrix is removed from the solution and rinsed in the MES buffer and allowed to air dry.

15 The binding characteristics of the boronated solid support matrix prepared as above using different soak times was measured using the elution assay described in Example 2. The resulting binding of total (open triangles) and glycated hemoglobin (closed triangles) is depicted in
20 Figure 1. Open circles show the ratio of glycated to total hemoglobin.

As more boronate groups are added to the solid support matrix, fewer carboxylic groups are left for ionic binding. The ratio of glycated hemoglobin to total hemoglobin
25 increases until there is a sufficiency of boronate groups, then the ratio remains the same even through the total binding is decreased.

Example 2Effect of Variable Incubation Times

5 An assay using the boronated support of Example 1 in a single measurement assay ("Single Measurement Method") was compared to a method of the present invention to determine the effect of variable incubation times on the concentration results obtained with each method.

10 The following assays were performed to compare the two methods. In the Single Measurement Method assay, blood lysate samples from a non-diabetic individual and a diabetic individual were diluted 1:1 with 500 mM ammonium acetate buffer pH=9.5 with 50 mM Mg^{++} and the samples were allowed to be in contact with the boronated solid support matrix prepared as in Example 1 for a variety of time periods. Following incubation, the boronated solid supports were rinsed with ammonium acetate buffer pH=9. Glycated hemoglobin was eluted with an elution buffer comprised of tris buffer at pH 8.0 containing 200mM sorbitol. The absorbance of the eluent was measured at 20 415nm.

25 In the second assay, corresponding to the method of the present invention, blood lysate samples from a non-diabetic individual and a diabetic individual were diluted 1:1 with mM MES buffer pH=6.5. The samples were allowed to be in contact with the boronated solid support matrix for time periods identical to those used for the first assay and were incubated at a similar time periods as done with the first assay. Following incubation the diluted samples were discarded and the boronated solid supports were rinsed 30 with MES buffer pH=6.5. The solid support matrixes were then rinsed a second time with 500 mM ammonium acetate

buffer pH=9.5 containing 50mM Mg^{++} . The absorbance of the
rinse was measured at 415nm corresponding to non-glycated
hemoglobin. Glycated hemoglobin was eluted off the matrix
and the absorbance of this rinse was measured. The data
5 obtained from the diabetic sample is provided in the Table
I (see also Figures 2 and 3).

Table I: Diabetic blood lysate sample

dwell time(min)	Present Invention			Single Measurement
	total	gly	Gly/total	Gly
0.25	0.769	0.12	0.156	0.029
0.5	0.739	0.109	0.147	0.032
1	0.824	0.118	0.143	0.037
2	0.846	0.116	0.137	0.038
4	0.838	0.123	0.147	0.047
8	0.856	0.126	0.147	0.072
16	0.814	0.125	0.154	0.082
4	0.779	0.118	0.151	0.049
8	0.844	0.103	0.122	0.064
16	0.857	0.121	0.141	0.082

10 Linear regression analysis of this data clearly showed
that the results obtained using the single measurement
method were heavily dependent on incubation time. However,
when using the methods of the present invention the results
obtained were independent of incubation time, which it is
15 believed will provide more reliable results and make the
assay more robust and suitable for untrained users.

Example 3

Effect of pH on Binding

The pH of the buffer affects the amount of hemoglobin
20 binding to the boronated support. If the pH is low, then

both glycated and non-glycated hemoglobin are bound. If the pH is high, only glycated hemoglobin will bind. Figure 4 depicts the binding of hemoglobin to the carboxy cellulose membrane (no boronate groups present) following the assay procedure described in Example 2. The membrane loses its ionic binding of hemoglobin between pH 6 and 7 when boronate is not present.

Figure 5 depicts the hemoglobin and human serum albumin binding properties of the carboxy cellulose support with added boronate groups, prepared as described in Example 1. Binding of the protein occurs at lower pH's due to the negatively charged carboxyl groups. The protein bound at the higher pH, due to the phenylboronate, is the glycated protein present in the samples.

Example 4

Description of Exemplary Strip Design Configurations

The boronated solid support matrices containing negatively charged groups can be incorporated in any assay where the separation of glycated from non-glycated protein is required. According to the preferred assay methods of the present invention the boronated solid support matrix is used in combination with other components to direct the flow of the sample and buffers. These components may be in the form of a strip which can be placed into a small, hand held reflectance meter for quantitation. Suitable alternative configurations for the strip are depicted in Figures 6A, 6B and 6C.

In the lateral flow strip configuration (Figure 6A), the fluids move through the length of the boronated support (parallel to the surface of the support). In the vertical flow (Figure 6B) and combination strip configuration

(Figure 6C), the movement of fluid is more through the thickness of the solid support matrix (perpendicular to the surface).

Each configuration depicted has a sample application
5 site (1, 11 or 21) which is a hole in a piece of plastic
(normally white for appearances), (4, 14 or 24). The
boronated solid support (2, 12 or 22) is in contact with
wicking material (6, 16 or 26). In the configuration
10 depicted in Figure 6A, fluids flow from the application
area (1) through the wicking material (6) to the support
(2) and through the support to the reservoir (3). In the
configurations depicted in Figures 6B and 6C, fluids flow
from the application area (11 or 21) and boronated support
(12 or 22) to the wicking material (16 or 26) and the
15 reservoir (13 or 23). All the components are placed on top
of the bottom piece of plastic, either white (15) or clear
(5 or 25) depending on the configuration. The reflectance
of the boronated support is measured by shining a light of
the selected wavelength onto the support surface and
20 measuring its intensity (7, 17 or 27). In the
configurations depicted in Figures 6B and 6C, there is a
piece of white reflecting material (18 or 28) placed behind
the support to reflect light passing through the support
back through it to reduce loss of intensity.

25

Example 5

Assay Proportionality: Glycated Hemoglobin

A vertical flow strip design described in Example 4
and as depicted in Figure 6B was used in assays of mixtures
of blood lysates from normal and diabetic blood samples.
30 Approximately 8 μ l of blood lysate was deposited on the
boronated solid support matrix prepared as in Example 1.

The application site was then rinsed with ~50 μ L MES buffer pH=6.5 (100 mM) and a reflectance measurement at 430 nm was recorded. The application site was then rinsed with ~50 μ L ammonium acetate buffer (500 mM) pH=9.5, containing 50mM

5 Mg⁺⁺ and a second reflectance measurement at 430 nm was recorded at the application site. The reflectance values were converted to K/S values which are proportional to the concentration of hemoglobin being measured. The data are shown in Table II below (see Figures 7 and 8).

10

Table II

norm.:diab.	%gly hb*	Reflectance		k/s		ratio(gly/total)
		Total	gly	Total	gly	
1:0	4.5	0.186	0.428	1.781	0.382	0.215
4:1	6.1	0.219	0.448	1.393	0.340	0.244
3:2	7.7	0.197	0.415	1.637	0.412	0.252
2:3	9.3	0.165	0.350	2.113	0.604	0.286
1:4	10.9	0.197	0.395	1.637	0.463	0.283
0:1	12.5	0.179	0.363	1.883	0.559	0.297

The regression statistics of the data plots are shown in the Table III below.

15

Table III

plot	Slope	intercept	S _{y.x}	CV*
Total Hb	0.031	1.48	0.255	14.7
Glycated Hb	0.026	0.24	0.076	15.9
Gly/total	0.010	0.18	0.010	3.8

*calculated using the mean values for each fraction

The plots, S_{y.x} and resulting C.V.'s show that the

20 total and glycated values measured vary from strip to strip. However, the large variations are reduced when the ratio of the two values is taken, demonstrating the advantage of using the methods of the present invention.

The results show that the calculated glycated fraction is proportional to the percent glycated hemoglobin in the sample.

The term CV is the coefficient of variation and is the measure of the scatter of replicate measurements around a mean value. The coefficient is calculated from the standard deviation ("SD") and the mean value ("M") of the replicates (i.e. $CV = 100 \times SD/M$). The benefit to using this value is that because it is expressed as a percentage it may be compared to other CV values without the requirement of knowing the mean values.

The term $S_{y.x}$ is a measure of variability used in linear regression calculation. It is the measure of the variability of "y" after removing the effect of "x" (i.e. it measures the variability of the data around the regression line).

Example 6

Assay Proportionality: Glycated Protein

A normal albumin sample was obtained from serum from a non-diabetic individual. An elevated glycated albumin sample was prepared from human serum albumin glycated *in vitro* (see, U.S. Pat. 5,589,393 to Michael D. Fiechtner, et al., issued December 31, 1996).

Both samples were assayed for fructosamine amount using a manual fructosamine assay based on Tietz, Textbook of Clinical Chemistry, 2nd Ed. (W.B. Saunders Co., Carl A. Burtis, Edward Ashwood, Eds., 1994), page 986-988. The elevated glycated albumin sample was found to contain 5.5 times the concentration of fructosamine as the normal albumin sample. The samples were mixed together in the proportions shown in Table IV (see also, Figures 9 and 10).

The resulting samples were assayed as described in Example 2 and the A280 absorbance values recorded above.

Table IV

Relative Fructosamine Conc.	measured total-gly	Measured glycated	calculated total	gly/total
1.0	0.065	0.027	0.092	0.029
1.0	0.067	0.028	0.095	0.029
2.1	0.056	0.037	0.093	0.040
2.1	0.052	0.037	0.089	0.042
3.3	0.045	0.058	0.103	0.056
3.3	0.049	0.045	0.094	0.048
4.4	0.040	0.053	0.093	0.057
4.4	0.038	0.054	0.092	0.059
5.5	0.033	0.063	0.096	0.066
5.5	0.030	0.072	0.102	0.071

5

Regression statistics for the above plot are given in the Table V below.

Table V

Fraction	slope	Intercept	R ²
total	0.001	0.091	0.177
glycated	0.009	0.020	0.908
gly/total	0.008	0.022	0.962

10 The calculated glycated albumin fraction was observed
to be proportional to the fructosamine content. The
results are proportional from the low sample concentration
to 5.5 times the low concentration as shown by the
regression statistics of R². Therefore the assay method
15 used to measure glycated hemoglobin is also suitable for
the measurement of glycated albumin.

Example 7Effect of Sample Volume

A lateral flow strip format as described in Example 4 and depicted in Figure 6A was used to assay non-diabetic and diabetic whole blood at varied sample volumes to determine the effect of sample volume on the assay, which followed the method described in Example 5. The measured absorbance values (415 nm) are presented in the Table VI (see Figures 11 to 12).

Table VI

absorbance (415)

μL sample	total Hb	Gly Hb	gly/total
10	1.164	0.183	0.157
20	1.053	0.167	0.159
30	1.233	0.164	0.133
40	1.206	0.195	0.162

The regression statistics are shown in the Table VII.

Table VII

Fraction	slope	Intercept	$S_{y.x}$	slope = 0?
Total Hb	0.0031	1.088	0.084	yes (P = 0.502)
gly Hb	0.0003	0.169	0.017	yes (P = 0.706)
Gly/total	-0.0001	0.156	0.016	yes (P = 0.893)

The results demonstrate that binding of total hemoglobin and glycated hemoglobin in the assay are independent of sample volume when the volume ranges from about 10 μL to about 40 μL in assays of whole blood.

Example 8Effect of Variation of Total Hemoglobin Concentration

The effect of varying the amount of total hemoglobin in the assay was determined using the assay procedure described in Example 2 to assay diluted blood samples. Table VIII below lists the absorbance values (415 nm) from the assays of non-diabetic and diabetic blood lysates diluted by factors of 2, 4, 8 and 16 (see Figures 13 to 15).

Table VIII

Sample	dil factor	A (415nm)		%gly
		Total Hb	gly	
Normal	2	1.309	0.091	6.9
	4	0.754	0.054	7.2
	8	0.382	0.026	6.8
	16	0.178	0.014	7.9
Diabetic	2	1.561	0.275	17.6
	4	0.929	0.157	16.9
	8	0.467	0.084	18.0
	16	0.199	0.036	18.1

The regression statistics are listed in the Table IX below for the graphs.

Table IX

sample	slope	Intercept	S _{y.x}	slope = 0?
normal	0.00060	0.067	0.0035	yes (P = 0.202)
Diabetic	0.00058	0.172	0.0049	yes (P = 0.329)

The amounts of total hemoglobin and glycated hemoglobin that bind to the solid support matrix decrease with decreasing total hemoglobin concentration. The calculated glycated hemoglobin/total hemoglobin, however, was constant over the dilutions assayed as seen in the

regression statistics (i.e. slopes are essentially zero).
This demonstrated the advantage of the methods of the
present invention (i.e. using the ratio of two measurements
to produce results independent of sample dilution or
5 hemoglobin concentration).

Example 9

Comparison to Other Commercial Assays

Twenty-five whole blood samples were assayed using the
strip configuration depicted in Figure 6C, and described in
10 Example 4 and following the assay method described in
Example 5. The samples were also assayed using two
commercially available tests for hemoglobin A1c.

The commercially available tests compared to the
method of the present invention were:

15 1. Roche Integra 400 (Roche Diagnostics)

The assay was performed by a desktop, multi-sample
analyzer using an immunoturbidimetric determination of the
glycated N-terminal valine of the β -chain in hemoglobin or
HbA1c according to the manufacturer's instructions. Each
20 sample was assayed once by this method.

2. DCA 2000 (Bayer)

The assay was performed by a desktop, single assay
analyzer using an immunoturbidimetric determination of
HbA1c according to the manufacturer's instructions. Each
25 sample was assayed once using this assay.

The results are plotted in Figures 16A and 16B and
show that the results obtained using the compared methods
and the methods of the present invention correlate.